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STUDIES ON THE HEPATOTOXICITY PRODUCED BY INTERACTION OF SEVERAL DRUGS CONTAINING AMINO GROUPS AND NITRITE

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ABSTRACT: *Hepatotoxicity study of the nitrosamine produced by interaction between drugs containing amino groups and soduim nitrite was conducted. In the in vitro study, interaction of 32mM Promethazine HCL or Oxomemazine HCL with 200mM Sodium nitrite produced N-nitroso dimetylamine (NDMA) at 0.4% or 0.03%, respectively. When sodium nitrite was administered with Promethazine HCL or Oxomemazine HCL, trace of NDMA was detected from the gastric contents. After three days of consecutive administration of sodium nitrite with Promethazine HCL or Oxomemazine HCL, the levels of GOT, GPT and SDH in the serum of treated groups were significant higher than that of control group.*

INTRODUCTION

Many nitrosamines are known to induce tumors on various organs (1,2). Recently it has been reported that there are a wide variety of drugs which contain tertiary amino groups in their molecules and these drugs interact with sodium nitrite to form N-nitrosodimethylamine (NDMA), a known carcinogenic nitroso compound formed in acidic conditions *in vitro* (3). Among them, aminopyrine and oxytetracycline were shown to form more reactive substances. Green vegetables contain a high amount of nitrite and the nitrite content in saliva was markedly elevated after ingestion of them (5,6). Nitrite is also used as a food additive. Therefore, it seemed important to examine a possibility of formation of nitrosamine by interaction between nitrite and several tertiary amino compounds *in vivo*. and *in vivo*. In the present study, after drug-nitrite interaction, we used TLC method to ascertain whether nitroso compound may be formed or not, and then examined a dose-response effect according to varying nitrite concentration by HPLC method *in vitro*. In addition we tried to determine the content of nitrosamine in the stomach by employing an HPLC and to evaluate the liver toxicity after a concommitant oral administration of amine drugs and nitrite.

MATERIALS AND METHODS

1. Experimental animals

Male Sprague-Dawley rats, weighing 200 ± 20g, were bred in the NIH animal room which was maintained at 23 ± 2°C and 55 ± 10% humidity with a 12hrs light-dark cycle. The animals were fed diet and water ad lihitum.

2. Chemicals and reagents

Aminopyrine(Dong-A Phrmaceutical Co.), promethazine HCl(Samsung Pharmaceutical Co.), oxomemazine HCl(Korean Rhone Poulenc Pharmaceutical Co), Orphenadrine HCl(Yoo Young Pharmaceutical Co.), N-nitrosodimethytamine and reduced β-nicotinamide dinucleotide(β-NADH) disodium salt are obtained from Sigma Chemical Company. Sodium nitrite, Triethanolamine, D(−)-fructose, sulphanilic acid, α-naphthylamine, hexane, diethylether, dichloromethane, chloroform, n-heptane and HPLC grade solvents (methanol and water) were obtained from the Japan Chemical Company.

3. TLC method for determination of nitrosamine

For a qualitative analysis of the nitrosamine formed after the interaction between the amine drug and nitrite *in vitro,* 10m*l* of the amine drug and nitrite mixture dissolved in diluted acetic acid solution(pH = 3) was incubated at 37 °C with shaking for 24 hours in dark condition. Final concentrations of aminopyrine, orphenadrine, promethazine and oxomemazine were 32mM and those of nitrite, 200mM. After incubation, reaction was stopped by the addition of solid NaOH and the mixture was extracted 3 times with 20m*l* of methylene chloride. The methylene chloride extracts were backextracted with 10m*l* of 1N HCl and evaporated and then dissolved in 0.5m*l* of methanol. This product was applied to the TLC plate(G60), developed in the solvent (chloroform:n-heptane:ethanol = 1 : 1 : 1), irradiated with short wave UV light (253nm) for about 3min and then sprayed with Griess reagent for visualization.

4. HPLC method for determination of nitrosamine

After the qualitative analysis of nitrosamine by TLC method, HPLC analysis was carried out to determine the relation between the yield of nitrosamine and the concentration of nitrite. Orphenadrine was ruled out because its Rf vaule was different from that of nitrosamine. Solutions of aminopyrine, promethazine and oxomemazine (32mM, respectively) containing variovs concentrations of nitrite(50, 100 and 200mM) were prepared and incubated at 37 °C in the dark with shaking for 1hour. The product which yielded by the same extraction method was diluted with 25m*l* methanol and analyzed by HPLC.

5. Evaluation of liver toxicity and HPLC determination of nitrosamine in the gastric contents after oral administration of amine drugs and nitrite

Male rats were transfered to individual cages for the interval feeding system (O′ hara Co., Ltd. Japan) and acclimatized until ambulation. The amounts of diet and water intake were constant. Solutions of aminopyrine, promethazine, oxomemazine and nitrite were freshly prepared in distilled water and administered intragastrically with an oral zonde after mixing one amine with nitrite. Sodium nitrite was administered at doses of 0.3, 0.6 and 1.2 mmole per kg body weight, equivalent to 20.7, 41.4 and 82.8mg per kg, respectively. Amine drugs were adminstered at a dose of 0.3 mmole per kg body weight. The dose-response effects of promethazine and oxomemazine were tested by their combined administration with three dosages of nitrite. Combined administration of aminopyrine with the highest dose of nitrite was used as the positive control. Rats given water, aminopyrine, promethazine, and nitrite served as concurrent controls. The compouds were administered intragastrically once a day for 3 consecutive days. The animals were anesthetized with ether at 30 min after the last dose and whole blood was obtained by cardiac puncture. Stomach was excised and its contents was washed with 5ml of distilled water and collected in the test tube. Serum was prepared by centrifugation. The activity of sGOT and sGPT were determined by a Bichromatic Analyzer (Abbott Vp series II) using a commercial diagnostic kit. The activity of serum sorbitol dehydrogenase was analyzed by a spectrophotometer (Pye Unicam sp 1750). The collected gastric contents were centrifuged and 1ml of their supernatant was extracted. The products were diluted to 1ml with methanol and analyzed by a HPLC under the same condition (Table 1).

RESULTS

1. Qualitative analysis of nitrosamine

TLC method was used to ascertain whether nitrosamines were produced after drug-nitrite interaction in vitro. A drug was incubated with nitrite for 24hr. Rf values of several products yielded in cases of promethazine, oxomemazine and aminopyrine was same as that of nitrosamine except for the orphenadrine.(Fig. 1).

Table. The codition of HPLC analysis

Column	u-Bondapak C18
Detector	U.V 254nm
Mobile phase	H20: methanol = 50:50
Flow rate	1ml per min
sensitivity	0.02
Column temperature	ambient
sample size	5ul

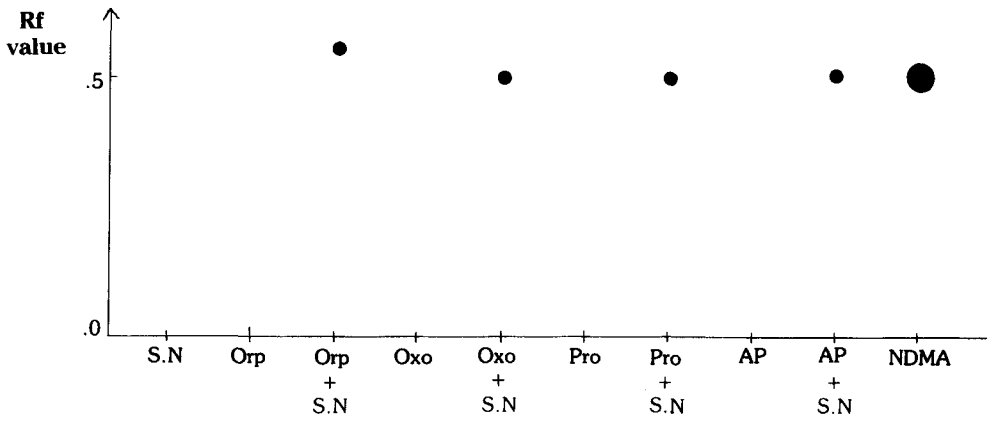

Fig. 1. Thin-layer chromatogram of reactive product formed from drugs. This figure was obtained by developing in a solvent mixture (chloroform: n-heptane: ethanol ± 1:1:1) and visualized with Griess reagent following irradiation of u.v light.

Table 2. The yield of N-nitrosodimethylamine *in vitro* after 1hr incubation.

Nitrite / Drugs (32mM)	Yield of N-nitrosodimethylamine (%) concentration of nitrite (mM) 50	100	200
Promethazine HCl	0.14	0.16	0.40
Oxomemazine HCl	0.006	0.012	0.03
Aminopyrine	N.E*	N.E	7.30

2. Quantitative analysis of nitrosamine

After the qualitation of nitrosamine by TLC method, it is confirmed that promethazine and oxomemazine have high possibility to produce nitrosamine after interaction with nitrite. Therefore, orphenadrine was ruled out for the determination of nitrosamine by HPLC method. The yield of NDMA produced after the drug-nitrite interaction *in vitro* with varying nitrite concentrations (50,100, and 200mM) were 0.14%, 0.16% and 0.4%, and, in the case of promethazine (32mM), it was and 0.006%, 0.012% and 0.03%, and, in the case of oxomemazine (32mM) and aminopyrine (32mM), which are well known to produce nitrosamine, they yielded 7.3% nitrosamine after interaction with 200mM nitrite (Table 2).

Determination of nitrosamine in gastric contents was carried out by HPLC method. Amounts of total NDMA produced were 11.56μg in case of aminopyrine. However, trace amounts of NDMA were detected by HPLC using UV deterctor at the highest nitrite concentration in cases of promethazine and oxomemazine.

Table 3. The effect of administration of promethazine HCl with sodium nitrite on biochemical parameters.

Drugs Promethazine (mmole/kg) Parameters (U/L)	Sodium nitrite (1.2)	Promethazine HCl (0.3)	Promethazine HCl (0.3) + Sod. nitrite (0.3)	Promethazine HCl (0.3) + Sod. nitrite (0.6)	Promethazine HCl (0.3) + Sod. nitrite (1.2)
GOT	110.1 ± 14.2	107.3 ± 13.7	145.1 ± 32.4	175.2 ± 25.7	162.8 ± 37.0
GPT	28.4 ± 5.6	29.5 ± 3.7	42.5 ± 7.8	74.3 ± 12.8	72.5 ± 20.3
SDH	N.D	N.D	4.2 ± 1.1	10.9 ± 4.4	12.0 ± 3.2

N.D; not detected

Table 4. The effect of administration of oxomemazine HCl with sodium nitrite on biochemical parameters.

Drugs (mmole/g) Parameters (U/L)	Sodium nitrite (1.2)	Oxomemazine HCl (0.3)	Oxomemazine HCl (0.3) ± Sod. nitrite (0.3)	Oxomemazine HCl (0.3) ± Sod. nitrite (0.6)	Oxomemazinee HCl (0.3)Sod. nitrite (1.2)
GOT	94.7 ± 7.1	91.7 ± 6.6	148.6 ± 16.2	156.7 ± 21.0	150.5 ± 27.5
GPT	26.4 ± 5.2	31.8 ± 4.8	51.2 ± 10.7	61.2 ± 12.7	60.7 ± 14.1
SDH	N.D	N.D	3.4 ± 1.4	5.7 ± 1.9	8.8 ± 3.2

N.D; not detected

3. Hepatotoxicity by the coadministration of drugs and nitrite

The alterations of several toxicological parameters, such as food and water consumption, ambulation, body weight change, glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase(GPT) and sorbitoal dehydrogenase(SDH) in sera were investigated for the purpose of semiquantitatively estimating the hepatotoxicity caused by nitrosamine produced after the interaction of drugs and nitrite in rats. Food and water consumption, ambulation and body weight were slightly reduced by coadministration of the drugs and nitrite. It seems that these parameters were not significantly different when compared to the control group within these short periods. Activities of GOT, GPT and SDH were significantly increased comparing to the concurrent control group when promethazine, oxomemazine and aminopyrine were orally administered with nitrite. However, dose-effect relatioship according to nitrite concentrations was not observed for GOT and GPT except for the SDH activity (Table 3, 4.).

These results were obtained from serum of rat sacrificed 30 min after last administration following oral administration of above drugs for three consecutive days. Number of animals was 4 in the single administration and 6 in the coadministration groups. All data were expressed as means ±standard deviation (M ± SD). Significance of dif-

ference between the two groups was analyzed by the unpaired 't' test. * ; $p < 0.05$, ** ; $p < 0.01$.

DISCUSSION

In the present experiment, main objective was to evaluate the potential nitrosability of several antihistamines during dissolution in the acidic condition.

At frist, we attempted TLC method as a screening test to ascertain the formation of nitrosamine *in vitro*. The suitable conditions for the interaction between drugs and nitrite were pH 3, temperature 37°C and 24 hours incubation time was selected; 24 hours in order to obtain a single and clear spot in the chromatogram. Dichloromethane was used as an extraction solvent. In the presence of dichloromethane, the protonated amine could complex with nitrite forming neutral complex which draws the nitrite ion into dichloromethane layer where nitrosamine formation can occur with great rapidity (8). In general, the nitrosamine is very unstable under the strong acidic environments or ultraviolet light (7). So, the extracted products were developed in the dark as quickly as possible. Rf values of products in cases of promethazine, oxomemazine and aminopyrine were same comparing to that of N-nitrosodimethylamine but that of orphenadrine is different.

And then HPLC analysis was carried out to estimate the relation between amounts of NDMA produced and concentration of nitrite. HPLC system using a uv detector has been introduced for the measurement of nitrosamine(8). When the amounts of nitro- of NDMA produced and concentration of nitrite. HPLC system using a UV detector has been widely used. But UV detector can be used when the amounts of products are enough to analyze. Nitrosamine formation was dependent on nitrite concentration in cases of promethazine and oxomemazine. The dependence of the production of nitrosamine on nitrite concentration has been reported (11). Degrees of nitrosamine formation from promethazine and oxomemazine at the high concentration of nitrite was 1/18 and 1/143 of aminopyrine, respectively. The possibility of nitrosation between drug containing tertiary amino group and nitrite in rat stomach was also investigated. Dosage schedule was selected on the basis of the amount of nitrosamine yield *in vitro* and acute toxic effect of NDMA(12, 13). Gastric contents were collected at 30 min after oral administration of drug and nitrite because gastric emptying time ranges 40 to 60 min (7) and 70 to 90% of nitrite in the stomach are obsorbed within 30 min(14). NDMA formed in the stomach vary between a trace to large amounts depending on gastric conditions, such as pH and food content. Nitrosamine detection from the gastric contents is very difficult by HPLC because gastric contents have various components such as protein and lipid that can disturb the detection of nitrosamine. Also gastric juice is known to contain various substances that may stimulate or inhibit the formation of nitrosamine. For exemple, thiocyanate is one of the potent catalysts of nitrosation. Although nitrosamine can also be formed in the stomach after drug-nitrite interaction, the dependency of nitrite concentration was not observed.

At last, serum enzyme activities such as GOT, GPT and SDH which reflect liver injury were investigated as biochemical parameters after 3 consecutive adminstration of amine and nitrite. Activities of GOT, GPT and SDH were significantly increased at any doses of nitrite concentration. But the dose-effect relationships were not consistent in cases of GOT and GPT except SDH. SDH was the most sensitive serum parameter in this study. These results coincide with the report that SDH is the most sensitive among serum enzymes in the evaluation of liver injury of the rat(12). The use of such biochemical and toxicological markers as indirect indices of *in vivo* biosynthesis of NDMA is more necessary than its chemical identification because of its rapid absorption and metabolism of nitrosamine.

From the above mentioned results, it is thought that N-nitrosodimethylamine can be produced *in vitro* and *in vivo* by the interaction of promethazine or oxomemazine with nitrite, and the produced N-nitrosodimethylamine can induce hepatotoxicity. However the significance of these findings with respect to man is not known since the toxicity resulting from interaction of amines and nitrite has not been investigated in humans. Therefore, it is suggested that further study to determine whether nitrosamine can be produced in man resulting from the ingestion of certain foods, which contains nitrite and nitrate, with secondary or tertiary amine at normal dietary levels.

CONCLUSION

It was found that when the drug containing a secondary or tertiary amino group reacts with sodium nitrite *in vitro* and *in vivo*, N-nitrosodimethylamine (NDMA) can be produced. It is well known fact that NDMA induces liver injury.

In the present study, we investigated that the nitrosability of three drugs which have a tertiary amino group (promethazine HCl, oxomemazine HCl and orphenadrine HCl) in the presence of sodium nitrite. To ascetain the formation of NDMA *in vitro* and *in vivo* as a result of coadministration of each drug and nitrite, TLC and HPLC method were carried out. And then we also investigated several toxicological parameters such as body weight and serum enzyme activity that reflect hepatic injury induced by NDMA. Experimental results are as follows:

1. Rf values on TLC plate in cases of promethazine HCl, oxomemazine HCl and aminopyrine(positive control) after the drug-nitrite interaction *in vitro* are same as that of NDMA except orphenadrine.
2. The yield of NDMA produced after the drug-nitrite interaction with varying nitrite concentrations (50, 100 and 200mM) were 0.14%, 0.16% and 0.4%, in case of promethazine HCl (32mM), 0.006%, 0.012% and 0.03%, in case of oxomemazine HCl, respectively, and, 7.3% in case of aminopyrine with 200mM nitrite. Maximal amounts (32mM) of NDMA can theoretically by produced.
3. Serum activities of GOT, GPT and SDH as parameters which reflect liver injury after the coadministration of amine drugs and nitrite were significantly increased comparing to the concurrent control group. But dose-effect relations of nitrite are not consistent in GOT and GPT except SDH.

4. Amounts of NDMA formed in the stomach after oral coadministration were only a trace in cases of promethazine HCl and oxomemazine HCl(0.3 mmole/kg), but 11.56ug in the case of aminopyrine (0.3mmole/kg).

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